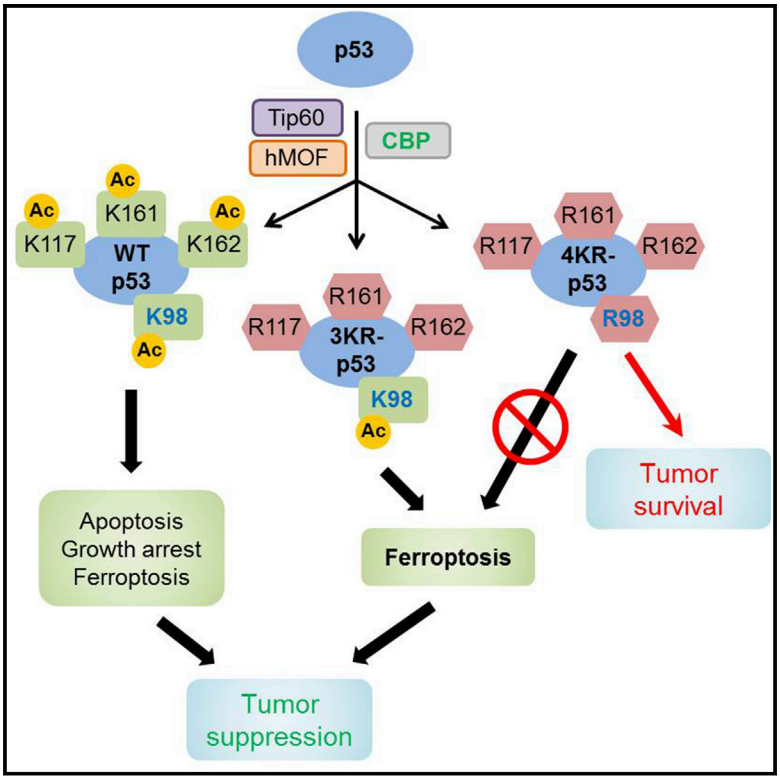
Report

Cell Reports

Acetylation Is Crucial for p53-Mediated Ferroptosis and Tumor Suppression

Graphical Abstract



Highlights

- CBP acetylates human p53 at K101 and mouse p53 at K98
- K98 acetylation of mouse p53 contributes to regulation of certain metabolic genes
- $p53^{4KR98}$ is defective in suppressing tumor growth
- Ferroptosis and repression of SLC7A11 are defective in $p53^{4KR98}$-expressing cells

Authors

Shang-Jui Wang, Dawei Li, Yang Ou, Le Jiang, Yue Chen, Yingming Zhao, Wei Gu

Correspondence

wg8@cumc.columbia.edu

In Brief

Wang et al. show that K98 acetylation of mouse p53 by CBP further contributes to the regulation of p53 transcriptional function by other known p53 acetylations (K117/161/162). Simultaneous absence of acetylation at K98 and at other positions in the DNA-binding domain results in the loss of tumor suppression in xenografts and ferroptosis.

Wang et al., 2016, Cell Reports *17*, 366–373
October 4, 2016
http://dx.doi.org/10.1016/j.celrep.2016.09.022

CellPress

OPEN ACCESS
CellPress

Cell Reports
Report

Acetylation Is Crucial for p53-Mediated Ferroptosis and Tumor Suppression

Shang-Jui Wang,[1,3] Dawei Li,[1,3] Yang Ou,[1] Le Jiang,[1] Yue Chen,[2,4] Yingming Zhao,[2] and Wei Gu[1,5,*]
[1]Institute for Cancer Genetics, Department of Pathology and Cell Biology, and Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University, 1130 Nicholas Avenue, New York, NY 10032, USA
[2]Ben May Department for Cancer Research, The University of Chicago, Chicago, IL 60637, USA
[3]Co-first author
[4]Present address: Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA
[5]Lead Contact
*Correspondence: wg8@cumc.columbia.edu
http://dx.doi.org/10.1016/j.celrep.2016.09.022

SUMMARY

Although previous studies indicate that loss of p53-mediated cell cycle arrest, apoptosis, and senescence does not completely abrogate its tumor suppression function, it is unclear how the remaining activities of p53 are regulated. Here, we have identified an acetylation site at lysine K98 in mouse p53 (or K101 for human p53). Whereas the loss of K98 acetylation ($p53^{K98R}$) alone has very modest effects on p53-mediated transactivation, simultaneous mutations at all four acetylation sites ($p53^{4KR}$: K98R+3KR[K117R+K161R+K162R]) completely abolish its ability to regulate metabolic targets, such as TIGAR and SLC7A11. Notably, in contrast to $p53^{3KR}$, $p53^{4KR}$ is severely defective in suppressing tumor growth in mouse xenograft models. Moreover, $p53^{4KR}$ is still capable of inducing the p53-Mdm2 feedback loop, but p53-dependent ferroptotic responses are markedly abrogated. Together, these data indicate the critical role of p53 acetylation in ferroptotic responses and its remaining tumor suppression activity.

INTRODUCTION

The p53 protein has been well characterized for its response to various cellular stresses, including induction of growth arrest, senescence, and apoptosis. Recently, however, roles of p53 have expanded beyond the canonical function of apoptosis and cell growth arrest and now include cellular processes, such as metabolism, oxidative balance, aging, autophagy, and ferroptosis (Feng et al., 2011; Jiang et al., 2015a; Levine and Oren, 2009; Vousden and Prives, 2009). Because of the extraordinary diversity of p53 functions, many have postulated that mechanisms exist to allow p53 to selectively activate downstream targets in specific functional groups, depending on the cellular context.

p53 activity is regulated by a complex network of fine-tuning mechanisms that include p53 protein stability, co-activator and inhibitor recruitment, and a diverse array of post-translational modifications, including acetylation, ubiquitination, phosphorylation, methylation, sumoylation, and neddylation (Eischen and Lozano, 2014; Gannon and Jones, 2012; Kruse and Gu, 2009). Specifically, acetylation of p53 has been established to play a major role in controlling promoter-specific activation of downstream targets during stress responses. Previous studies have demonstrated that acetylation at K120 by Tip60/MOF is important for p53-mediated apoptosis (Sykes et al., 2006; Tang et al., 2006), whereas acetylation at K164 by CBP/p300, along with K120 acetylation, contributes to p53-induced cell cycle arrest (Tang et al., 2008). More recently, the $p53^{3KR}$ mouse model that expresses acetylation-deficient p53 (K117/161/162R), which mirrors the K120/164R mutations in human p53, demonstrated that, whereas apoptotic and growth arrest functions of p53 are lost, p53-dependent metabolic regulation is still intact (Li et al., 2012).

Although the molecular mechanism of how p53 achieves tumor suppression is not entirely understood, it has long been accepted that tumor proliferation can be inhibited by p53-mediated apoptosis, cell growth arrest, and senescence. However, several mouse models suggest that tumor suppression by p53 can be achieved in the absence of those canonical functions. Analysis of the p21/WAF1 knockout mice revealed no evidence of spontaneous tumorigenesis in the absence of growth arrest (Choudhury et al., 2007). Similarly, studies on apoptosis-deficient Puma and Noxa double knockout mice also failed to observe any increased tendency in tumor formation (Michalak et al., 2008). More strikingly, the $p53^{3KR}$ acetylation-deficient and the $p53^{25,26}$ TAD mutant mouse models, both of which lack the ability to undergo p53-mediated apoptosis and cell cycle arrest, are not significantly prone to developing tumors when compared to wild-type mice (Brady et al., 2011; Li et al., 2012). Data from the $p21^{-/-}puma^{-/-}noxa^{-/-}$ triple knockout mice further validated the notion that apoptosis and growth arrest are dispensable for tumor suppression (Valente et al., 2013). Furthermore, our most-recent study showed that, whereas $p53^{3KR}$ cannot elicit cellular apoptosis, it retains the ability to promote ferroptosis, an iron-dependent alternative

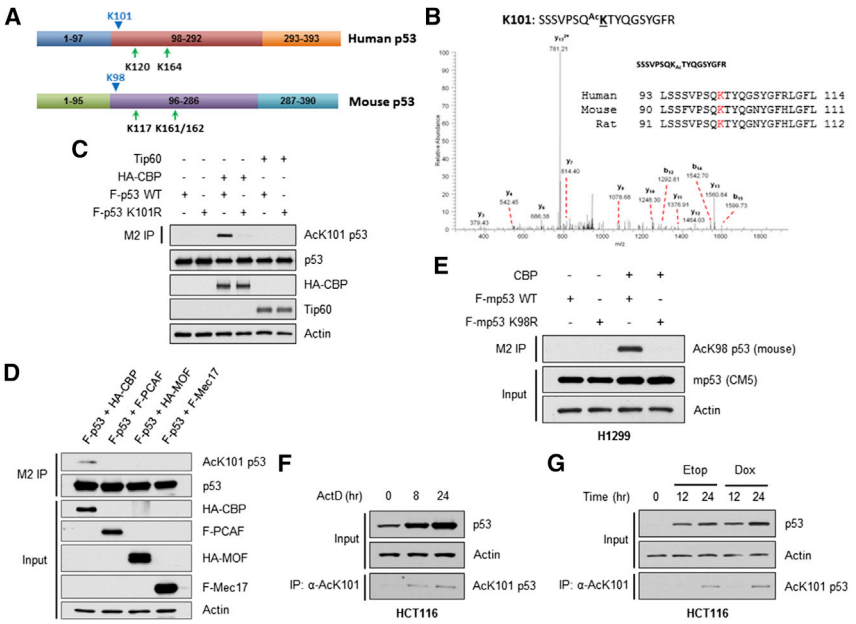


Figure 1. Acetylation of Human p53 at K101 and Mouse K98 by CBP

(A) Cartoon figure depicting the K101 lysine residue (K98 for mouse p53) located in the N-terminal end of the DNA-binding domain.

(B) Mass spectrometry of purified p53 protein in the presence of CBP uncovered acetylation of p53 at the K101 lysine residue.

(C) H1299 cells were transfected with vectors expressing either Flag-tagged human wild-type or K101R mutant p53 in the presence of CBP and Tip60. Whole-cell lysates were collected, and Flag-tagged p53 was immunoprecipitated using M2 Flag beads. K101-acetylated p53 was detected using site-specific antibody. See also Figures S1 and S3A.

(D) H1299 cells were transfected with vectors expressing Flag-tagged human wild-type p53 and various acetyltransferases (CBP, PCAF, MOF, and Mec17). Similar to (C), site-specific acetylation antibody against AcK101-p53 was used to detect presence of K101 p53 acetylation after immunoprecipitation of p53. See also Figure S2.

(E) Similar experiment to (C) was performed using Flag-tagged mouse wild-type or K98R mutant p53 in the presence of CBP, and p53 acetylation at K98 was detected using the same site-specific antibody against acetylated K101.

(F) HCT116 cells were treated with 10 nM actinomycin D for 0, 8, and 24 hr. K101-acetylated p53 was immunoprecipitated using site-specific antibody from whole-cell lysates, and the immunoprecipitation (IP) products were detected using DO-1 (p53) antibody. See also Figure S3B.

(G) Similar experiment to (F) was performed using 20 μM etoposide and 0.2 μg/mL doxorubicin treatments for 0, 12, and 24 hr.

cell death pathway, which may constitute a potential mechanism of p53-mediated tumor suppression (Jiang et al., 2015a).

To further elucidate the mechanism of p53 regulation, we conducted a mass spectrometry screening to uncover previously unknown modifications of p53. Here, we identified a p53 acetylation site at lysine K101 within the DNA-binding domain. Lysine residue K101 of p53 is evolutionarily conserved and has been found to be mutated in human cancers. In this study, we demonstrated that K101 in human p53, as well as the homologous K98 lysine residue in mouse p53, can be acetylated by acetyltransferase CBP. Acetylation at this site does not contribute to p53 stability or DNA-binding capabilities. Ablation of K98 acetylation in mouse p53 alone only resulted in a modest decrease in the transcriptional activity of p53. However, simultaneous loss of K98 acetylation ($p53^{4KR98}$) with the previously characterized K117/161/162 acetylations ($p53^{3KR}$) significantly abrogates p53-mediated transcriptional regulation of its metabolic targets, including TIGAR, GLS2, and SLC7A11. Interestingly, $p53^{4KR98}$ is unable to induce ferroptosis, and its ability to thwart cancer growth is also abrogated. Thus, our studies have significant implication regarding the role of acetylation in regulating p53-mediated ferroptosis and tumor suppression.

RESULTS

p53 Is Acetylated at the K98 Lysine Residue by CBP

To screen for unidentified acetylation of p53, we purified and analyzed ectopically expressed p53 protein in H1299 cells in the presence of different histone acetyltransferases by mass spectrometry. To increase the yield of acetylated forms of p53, we treated the cells with deacetylases inhibitors trichostatin A (TSA) and nicotinamide for 8 hr before isolating the acetylated p53. Our mass spectrometry data showed that, in the presence of acetyltransferase CBP, p53 is found to be acetylated at lysine residue K101, a previously uncharacterized p53 modification (Figures 1A and 1B). CBP is a histone acetyltransferase that also acetylates p53 at K164 in the DNA-binding domain (Tang et al., 2008). The K101 lysine residue resides in the DNA-binding domain of p53 and is homologous to the K98 lysine residue in mouse p53 (Figure 1A). Interestingly, the K101 lysine residue appears to be evolutionarily conserved (Figure 1B).

To validate the acetylation at K101, we generated a rabbit monoclonal antibody specifically against p53 acetylated at lysine K101. We first confirmed p53 acetylation by CBP by transfecting H1299 p53-null cells with vectors expressing either Flag-tagged wild-type p53 or K101R p53 in the absence or presence of acetyltransferase CBP, and the tagged p53 were immunoprecipitated and resolved on SDS-PAGE for western blot analysis. The site-specific AcK101-p53 antibody only recognized wild-type p53 acetylated by CBP, but not acetylation-deficient K101R p53 mutant (Figure 1C, lanes 1–4), which confirmed the mass spectrometry data that CBP indeed catalyzes the acetylation of p53 at K101. In vitro acetylation assay using purified p53 and CBP proteins further validated our in vivo findings (Figure S1). Interestingly, Tip60, an acetyltransferase that acetylates p53 at K120 lysine residue in the DNA-binding domain, does not acetylate p53 at K101 (Figure 1C, lanes 5 and 6). Furthermore, co-transfection of vectors expressing other acetyltransferases (PCAF, MOF, and Mec17) with p53-expressing vectors did not result in K101 acetylation of p53 (Figure 1D). However, given its structural similarity to CBP, p300 is able to acetylate p53 at the K101 lysine residue as well, albeit with significantly lower

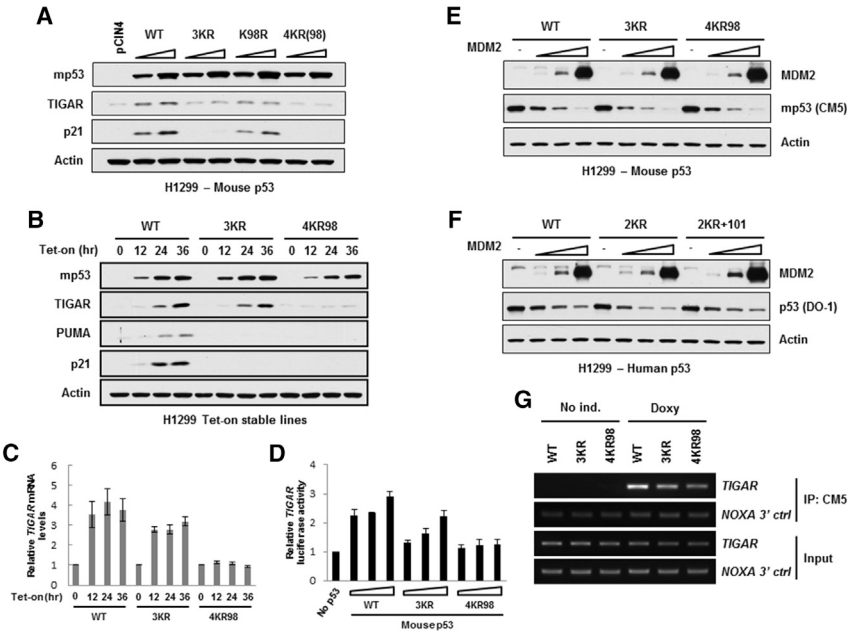


Figure 2. Effect of K98 Acetylation on the Activation of *TIGAR* Gene, p53 Stability, and DNA Binding Capacity

(A) H1299 cells were transfected with empty vector and vectors expressing wild-type and various KR mutant p53. Expressions of TIGAR and p21 were detected via western blotting.

(B) H1299-inducible p53 stable lines expressing wild-type, 3KR, or 4KR98 p53 were treated with 5 μg/mL doxycycline for 0, 12, 24, and 36 hr to induce p53 expression. Expressions of p53 targets were detected via western blotting using TIGAR, PUMA, and p21 antibodies.

(C) Cells were treated similar to (B), total RNA was extracted and reverse transcribed to cDNA, and qPCR was performed to measure *TIGAR* mRNA levels. Bar graph depicts mean ± SEM of three separate experiments. See also Figure S4.

(D) H1299 cells were transfected with *TIGAR* luciferase construct along with increasing amount of plasmids expressing wild-type, 3KR, or 4KR98 p53. Cell lysates were collected and assayed for luciferase activity. Bar graph depicts mean ± SEM of three separate experiments.

(E and F) H1299 cells were transfected with vectors expressing either (E) human p53 or (F) mouse p53 in the presence of increasing amount of MDM2-expressing plasmid. The levels of MDM2 and p53 proteins were determined via western blotting using MDM2 and DO-1 (human p53) or CM5 (mouse p53) antibodies.

(G) ChIP assay was performed on Tet-on-inducible H1299 cells that were treated with doxycycline to induce p53 expression for 8 hr. The immunoprecipitated DNA was amplified by PCR using primers that span the p53-binding region on *TIGAR* promoter, and the PCR products were resolved on agarose gel.

efficiency (Figure S2). A similar experiment was performed using mouse p53, and the AcK101-p53 antibody also recognized acetylation of mouse p53 at K98 in the presence of CBP (Figure 1E). Moreover, we further validated the specificity of the AcK101-p53 antibody against p53 with acetylated K101 residue by demonstrating the inability of the antibody to immunoprecipitate the acetylation-deficient $p53^{K101R}$ mutant (Figure S3A).

Next, we wish to investigate whether p53 acetylation at K101 occurs endogenously. AcK101-p53 antibody was used to immunoprecipitate the K101-acetylated form of p53 in HCT116 cells after DNA damage, and the levels of AcK101-p53 were detected using anti-p53 DO-1 antibody. As seen in Figures 1F and 1G, the steady-state levels of AcK101-p53 increased as p53 became stabilized after drug treatments. Knockdown of CBP in HCT116 cells significantly diminished the level of K101 acetylation after DNA damage, although trace amount of acetylation can still be detected, likely due to overlapping acetyltransferase activities from p300 (Figure S3B; also see Figure S2). Together, our results indicate that K101 lysine residue of p53 is a bona fide acetylation site of CBP both in vitro and in vivo.

Simultaneous Mutations at K117/161/162 and K98 Impair p53 Transcriptional Activities

Previous studies have shown that $p53^{3KR}$ with triple lysine-to-arginine mutations K117/161/162R retains regulation on *Mdm2* and certain metabolic targets (Li et al., 2012). In light of this, we wish to investigate whether ablation of K98 acetylation would further impair mouse p53 transcriptional activities. Ectopic expression of mouse $p53^{K98R}$ in H1299 p53-null cells showed that the transcriptional activity of this single-mutation p53 was comparable to wild-type p53, with only a subtle decrease in p21 and TIGAR transactivation. However, when simultaneously mutating lysine residues at K117/161/162 and K98, the resulting mouse $p53^{4KR98}$ mutant exhibits significant defect in transactivating TIGAR expression (Figure 2A). To characterize the dynamics of downstream target expression in a more-physiological manner, Tet-on-inducible H1299 stable lines conditionally expressing wild-type mouse p53 and various mouse p53 mutants were generated. Induction of wild-type p53 and $p53^{3KR}$ expression by doxycycline treatment led to increased expression of TIGAR, whereas induction of the $p53^{4KR98}$ mutant failed to do so (Figure 2B). These findings demonstrate that ablation of the K98 acetylation alone does not significantly affect mouse p53 activity, suggesting functional redundancy through other acetylations (such as acetylations at K117/161/162). However, disrupting all four acetylation sites exhibits significant defect in p53 transcriptional activity, which indicates that, in the absence of K117/161/162 acetylations, K98 acetylation may be critical for p53-mediated regulation on certain targets.

To confirm that the defect in activating downstream targets by $p53^{4KR98}$ is transcriptional in nature, we quantified the mRNA levels of *TIGAR* after p53 induction in Tet-on-inducible stable lines. Indeed, induction of wild-type mouse p53 and $p53^{3KR}$ enhanced *TIGAR* mRNA expression, whereas induction of $p53^{4KR98}$ failed to do so (Figure 2C). Similarly, co-transfection of *TIGAR* luciferase reporter construct with vector expressing the $p53^{4KR98}$ mutant in H1299 p53-null cells resulted in diminished reporter activity, compared to transfection of vectors expressing wild-type mouse p53 and $p53^{3KR}$ mutant (Figure 2D). In addition, consistent with our previous data, transcriptional

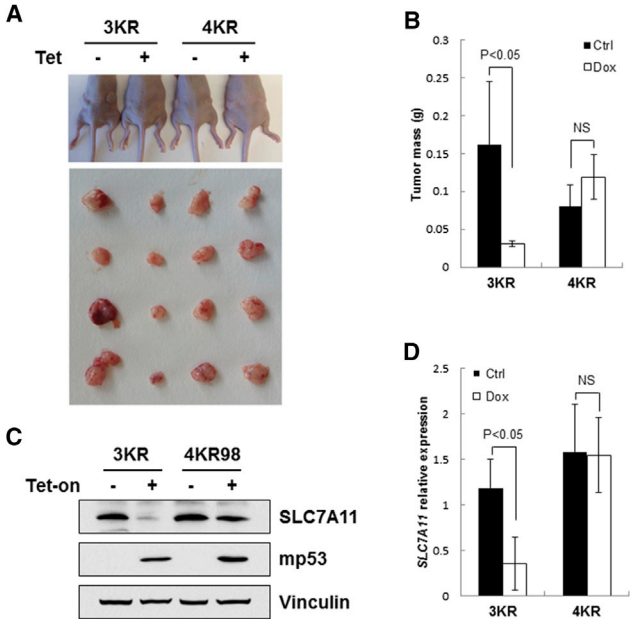


Figure 3. Effect of K98 Acetylation on Tumorigenicity

(A) Xenograft tumors obtained from Tet-on p53 3KR and 4KR98 cells that were injected into the flanks of Nude mice.

(B) Tumor weights were determined (mean ± SEM from four tumors). Independent experiments were repeated two times, and representative data are shown.

(C and D) SLC7A11 levels in tumor tissues were determined by (C) protein expression via western blot and (D) mRNA quantification via qPCR analysis (mean ± SEM from four tumors).

regulation on *GLS2* is retained by $p53^{3KR}$, but not by $p53^{4KR98}$ (Figure S4).

Acetylation in the DNA-Binding Domain Does Not Affect p53 Stability or DNA Binding

One of the functions of p53 acetylation is to increase p53 stability by competing against ubiquitination on lysine residues. Therefore, we wish to examine whether or not mouse K98 and other acetylation sites in the DNA-binding domain affect p53 stability. We observed that, in the presence of human MDM2, wild-type mouse p53 and the lysine-to-arginine mouse p53 mutants are degraded to a similar degree (Figure 2E). Similar results were obtained using human p53, suggesting that acetylations in the DNA-binding domain do not affect p53 stability (Figure 2F).

Acetylation in the DNA-binding domain also raises the question of whether these acetylations alter the affinity of p53 to DNA. To assess the DNA-binding capabilities of mouse p53 with different acetylation potentials, we performed chromatin immunoprecipitation (ChIP) using wild-type and various acetylation-deficient mouse p53. After doxycycline-induced p53 expression in the Tet-on-inducible cell lines, wild-type mouse p53 and $p53^{3KR}$/$p53^{4KR98}$ mutants were all able to recruit to the *TIGAR* gene promoter, albeit with a slight reduction in binding affinity with the $p53^{4KR98}$ mutant (Figure 2G). However, the TIGAR expression is completely abrogated in the presence of the $p53^{4KR98}$ mutant (Figures 2A–2C), suggesting that its mild effect on DNA binding could not account for the transcriptional defect observed. These data indicate that the acetylation in the DNA-binding domain does not dramatically affect p53 stability or DNA binding to exert control on p53 transcriptional function.

Loss of K98 Acetylation Impairs the Tumor-Suppressive Function of p53

As clearly demonstrated by the $p53^{3KR}$ knockin mice, tumor-suppressive activity of $p53^{3KR}$ is largely preserved. Although $p53^{3KR}$ maintains its transcriptional regulation on a subset of p53-mediated targets, which include *MDM2* and several metabolic genes (i.e., *TIGAR* and *GLS2*), the downstream effector(s) that confer the observed tumor suppression by $p53^{3KR}$ is still unknown. Whereas metabolic targets, such as TIGAR and GLS2, are postulated to play a role in p53-mediated tumor suppression, they may have dual opposing roles of preventing cancerous metabolic alterations on the one hand and tumor survival on the other (Wang and Gu, 2014). Nevertheless, we decided to investigate whether or not K98 acetylation would contribute to the tumor suppressor function of p53. To test this, we performed xenograft assays by injecting Tet-inducible p53-null H1299 cells expressing $p53^{3KR}$ or $p53^{4KR98}$ in Nu/Nu Nude mice (Charles River Laboratories). Upon tetracycline induction of $p53^{3KR}$, the growth of injected H1299 cells was significantly diminished in the xenograft assays; surprisingly, however, induction of $p53^{4KR98}$ in H1299 cells resulted in no reduction of xenograft growth (Figures 3A and 3B).

Even though our data demonstrated that TIGAR expression is lost in $p53^{4KR98}$, recent study with *Tigar*-knockout mice suggested that TIGAR function actually promotes efficient tumorigenesis through reactive oxygen species (ROS) regulation (Cheung et al., 2013) and therefore is unlikely to contribute to the loss of tumor suppression in $p53^{4KR98}$. Because our lab has recently linked SLC7A11-dependent ferroptosis to p53-mediated tumor suppression (Jiang et al., 2015a), we decided to investigate the expression of SLC7A11 in the xenograft tumors. SLC7A11 plays a crucial role in cystine uptake that subsequently prevents ferroptosis, and downregulation of SLC7A11 expression through transcriptional regulation by p53 can induce this iron-dependent cell death process independently from apoptosis and therefore represents a potentially significant conduit that exerts p53-mediated tumor suppression. As expected, induction of $p53^{3KR}$ repressed the levels of SLC7A11 protein and mRNA in xenograft tumors; however, to our surprise, SLC7A11 expression in xenografts remained robust in the presence of $p53^{4KR98}$ activation (Figures 3C and 3D).

Regulation of SLC7A11 Gene Expression and Ferroptosis Response Is Retained by $p53^{3KR}$ but Lost in $p53^{4KR98}$

We further validated the effect of K98 acetylation on the regulation of *SLC7A11* gene using H1299 Tet-on-inducible cell lines. As consistent with the xenograft data, the protein levels of SLC7A11 were significantly reduced in the presence of wild-type p53 and $p53^{3KR}$, whereas no effect on SLC7A11 expression was observed with $p53^{4KR98}$ protein expression (Figure 4A). Protein levels of p21, PUMA, TIGAR, and MDM2 reflect the observed transcriptional function of the respective wild-type

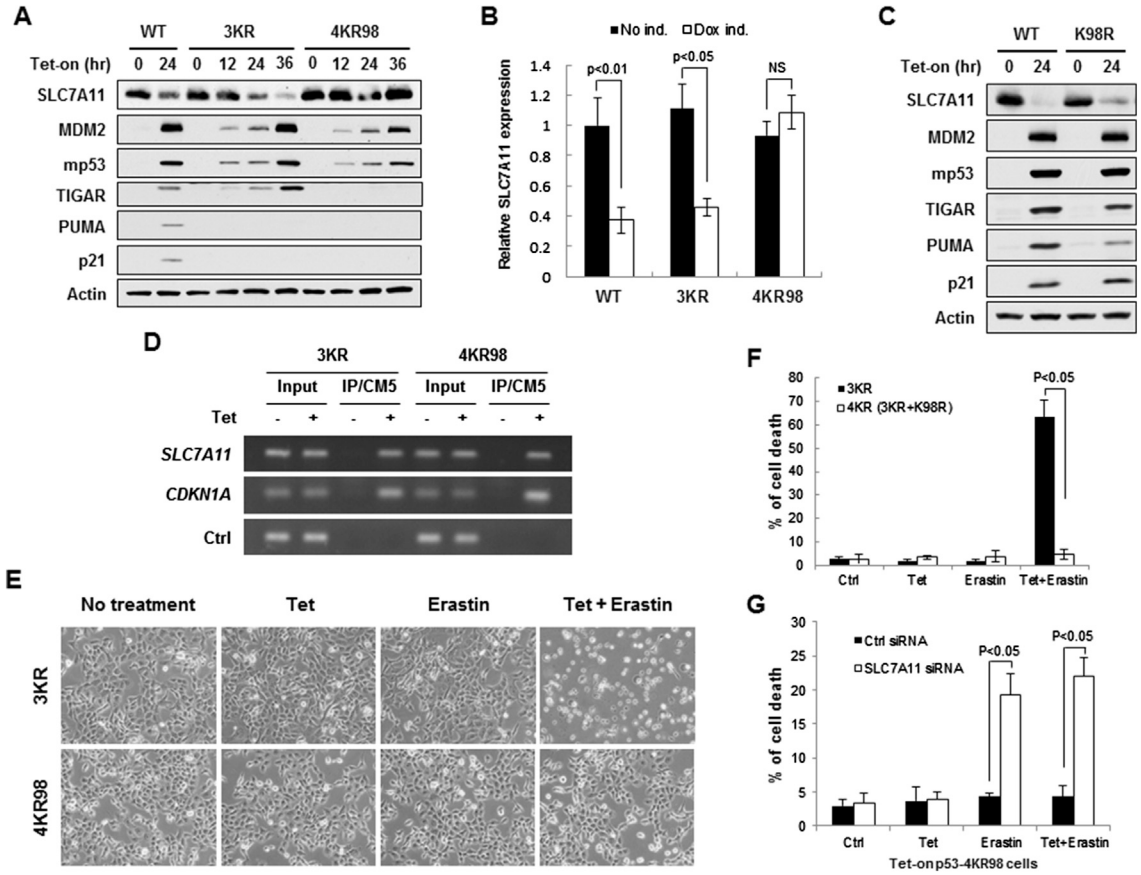


Figure 4. Regulation of *SLC7A11* Gene Expression and p53-Dependent Ferroptosis Are Retained by $p53^{3KR}$, but Not by $p53^{4KR98}$

(A) Tet-on-inducible H1299 cell lines were induced with doxycycline for the length of times indicated, and crude cell lysates were obtained for western blot analysis using antibodies against SLC7A11, MDM2, TIGAR, PUMA, p21, p53, and actin.

(B) Total RNA was extracted from Tet-on-inducible H1299 cell lines that were either not induced or induced with doxycycline for 24 hr. qPCR was performed to measure the mRNA levels of *SLC7A11*. Bar graph depicts mean ± SEM of three separate experiments.

(C) Expression profile of Tet-on-inducible H1299 cell lines expressing wild-type and K98R p53 was analyzed similar to (A).

(D) ChIP assay was performed on Tet-on-inducible H1299 cells conditionally expressing 3KR and 4KR98 p53 that were treated with doxycycline to induce p53 expression for 8 hr. The immunoprecipitated DNA was amplified by PCR using primers on *SLC7A11* and *CDKN1A* (p21) promoters, and the PCR products were resolved on agarose gel.

(E) Tet-on-inducible H1299 cells conditionally expressing 3KR or 4KR98 p53 were treated with doxycycline, erastin, or doxycycline + erastin for 48 hr, at which cell death was observed.

(F) The relative number of live and dead cells was counted for (E), and the graph depicts the mean ± SEM of three separate experiments.

(G) Tet-inducible H1299 cells expressing 4KR98 p53 were subjected to either mock or SLC7A11 depletion via small interfering RNA (siRNA) for 48 hr and were either left untreated or subsequently treated with doxycycline, erastin, or both for 36 hr. Cell counting was done with trypan blue via hemocytometer. Percent dead cells was calculated and reported in the bar graph, which depicts the mean ± SEM of three separate experiments.

See also Figure S5.

and mutant p53. Similarly, mRNA expression of *SLC7A11* upon wild-type p53 and $p53^{3KR}$ induction both reduced to ~40% of baseline, whereas its expression after $p53^{4KR98}$ induction remained unchanged (Figure 4B). As expected, repression of SLC7A11 expression level was only minimally affected by the absence of K98 acetylation (Figure 4C). Interestingly, although $p53^{4KR98}$ mutant has lost its ability to repress SLC7A11 expression, it still retains its ability to bind to the *SLC7A11* promoter (Figure 4D).

SLC7A11 gene codes for a subunit of the cystine/glutamate antiporter (system x_c^-), which antagonizes one of the pathways that leads to ferroptosis. This process is inhibited by erastin, an inhibitor of system x_c^-, and consequently promotes ferroptosis through upstream blockade of cystine uptake (Dixon et al., 2012). H1299 cells, like many other cancer cells, have elevated levels of SLC7A11 expression and therefore are resistant to erastin-induced ferroptosis in the absence of p53 induction (Figure 4E, third panel from the left). However, in the presence of $p53^{3KR}$ expression after doxycycline induction, repression of SLC7A11 expression by p53 sensitized cells to undergo erastin-induced ferroptosis, whereas $p53^{4KR98}$ induction failed to do so (Figure 4E, right-most panel). Quantification of cell death and survival is shown in Figure 4F. Moreover, SLC7A11 depletion in Tet-inducible H1299 cells expressing $p53^{4KR98}$ reversed the

ferroptotic-resistant phenotype of $p53^{4KR98}$-expressing cells after erastin treatment (Figures 4G, S5A, and S5B). A recent study has also implicated GLS2, a known p53 metabolic target, as an inducer of ferroptotic cell death by way of promoting glutaminolysis (Gao et al., 2015). As shown earlier, $p53^{4KR98}$ also failed to induce *GLS2* expression, whereas $p53^{3KR}$ still retained regulation on *GLS2* (consistent with our prior study) and thus may also contribute to the inability of $p53^{4KR98}$ to elicit ferroptotic response (Figure S4). Together, these results indicate that K98 acetylation of mouse p53 plays a critical role in the repression of SLC7A11 gene expression and p53-mediated ferroptosis, which may contribute to the observed suppression of tumor growth in our xenograft model.

DISCUSSION

The role of p53 acetylation can be thought of as a "code" that dictates the functional purpose of p53 by allowing a specific subset of downstream targets to be regulated by the p53 complex that possesses the corresponding code, which is represented by the combination of the acetylated lysine residues. We observed that ablation of acetylation at K98 in mouse p53 alone did not affect transcriptional activity of p53. Interestingly, when simultaneously disrupting acetylations at K117/161/162 and K98, transactivation of *TIGAR* gene and repression of *SLC7A11/Slc7a11* gene, which are retained by $p53^{3KR}$, were compromised. Collectively with previous findings, our data suggest that the acetylations in the DNA-binding domain of p53 may have redundant roles in promoter-specific regulation of p53 metabolic targets, such as *TIGAR* and *SLC7A11/Slc7a11*. In other words, the acetylation codes for regulating p53 metabolic targets are many and complex in nature; various acetylation statuses of p53 could permit such regulatory capacity, although it is unclear whether there are differences in the effectiveness of each acetylation code or disparity in the degree and context of occurrences for each acetylation code. In mark contrast, the p53 acetylation code for apoptotic activation via *Puma/Noxa/Bax* is unique, as it simply requires site-specific acetylation at K117, and loss of K117 acetylation completely abrogates p53-mediated apoptotic response (Li et al., 2012). The acetylation requirement for p53-mediated growth arrest lies somewhere in between metabolic and apoptotic regulation, in which acetylation at either K117 or K161/162 is sufficient to elicit p21 activation and resulting G1/S-phase arrest (Li et al., 2012). Interestingly, our data indicate that p53 regulation on *MDM2* appears to be retained by $p53^{4KR98}$, suggesting that there is even a lesser p53 post-translational requirement for *MDM2* regulation.

Whereas it is unclear how the upstream signaling pathways and mechanics lead to specific p53 acetylations to bring about the desired p53 function in any given cellular context, it is evident that the regulation and manipulation of p53 acetylation has significant biological consequences (Mellert and McMahon, 2009; Zhang et al., 2012a, 2012b). Nevertheless, our current understanding of differential regulation through acetylation poses interesting speculation of its biological purpose. In the absence of cellular stress, K98 acetylation in mouse p53 may be sufficient for p53-mediated regulation of genes, such as *Tigar* or *Slc7a11*. Functionally, expression of TIGAR under non-stressed condition is appropriate, as TIGAR activity may contribute to normal cellular energy and ROS homeostasis. Because K98 acetylation is not sufficient to induce growth arrest and apoptotic targets, it is a harmless form of p53 activation that can occur in the absence of stress. Repression of SLC7A11 expression alone via p53 is also non-toxic, as expression of $p53^{3KR}$ in cell lines, mouse embryonic fibroblast (MEF) cells, or in mice does not trigger ferroptosis. Induction of ferroptosis requires additional cellular insults (i.e., ROS stress or erastin exposure) in the setting of p53-mediated SLC7A11 repression (Jiang et al., 2015a, 2015b). As such, downregulation of SLC7A11 under basal p53 regulation or low stress condition may provide another layer of protection against tumorigenesis by decreasing the threshold in which cells can tolerate ROS stress and allowing activation of programmed cell death to circumvent potential harm from genetic instability. As cells encounter genotoxic stress, p53 may determine cell fate depending on the stress level (Helton and Chen, 2007), and the decision making may be exerted by distinct p53 acetylations. With mild stress, K161/162 lysine residues can be acetylated to induce growth arrest (without triggering apoptosis) via p21 activation, which allows cells to repair and recover from cellular damage. However, in the face of severe stress that causes irreparable cellular damage, mouse p53 acetylation at K117 will prompt an apoptotic response through expression of PUMA/NOXA/BAX, leading to self-elimination to prevent the potential propagation of deleterious mutations. All the while, regulation of MDM2, a key moderator of the highly dynamic p53, prevails in the absence of all the above-mentioned acetylations, underscoring the indispensable role of the negative-feedback loop for maintaining a functional p53 axis.

Even though the acetylation sites in the DNA-binding domain play crucial roles in p53 activation and differential regulation, our data suggest that they do not appear to significantly affect promoter-specific binding. For example, we observed that wild-type p53, $p53^{3KR}$, and $p53^{4KR98}$ can all bind *TIGAR* gene promoter, yet these p53 with varying capacity in acetylation possess drastically different transcriptional activity on *TIGAR*. One possibility is that the acetylations in the DNA-binding domain alter the conformation of the p53 and in turn influence co-activator or transcriptional machinery recruitment instead of affecting DNA binding. Co-activators, usually histone acetyltransferases (such as CBP/p300, PCAF, GCN5, and Tip60), are recruited by transcription factors to acetylate histones and relieve the coiling of chromatin to allow transcription to occur. Thus, it is possible that different acetylations or combinations of acetylations may present as a form of code for association with various co-activators and therefore allow different p53 targets to be expressed depending on the co-activator present. Testing whether different co-activators are recruited based on the presence of p53 modifications in a promoter-specific manner could shed light on the mechanism behind differential regulation by p53.

It is interesting to note that, even though there are documented mutations of the K101 lysine residue in human cancer, the prevalence is low. Per the International Agency for Research on Cancer (IARC) TP53 database for human p53 somatic mutations (n = 24,320), there are only eight mutations documented for K101, compared to 1,324 mutations for the R175 hotspot that

completely abolish p53 transcriptional function (http://p53.iarc.fr/). Given the implications derived from our study, this does not come as a surprise, as mutation of the homologous K98 lysine residue alone in murine p53 did not result in significant functional impairment. Only in the absence of other acetylations in the DNA-binding domain does the status of K98 acetylation become of paramount importance. Nevertheless, our study underscores the mechanistic significance of p53 acetylation in exerting tumor suppression and sheds light on the long-standing question of how p53 may intelligently control a vast multitude of targets as a master regulator.

The $p53^{3KR}$ mouse model, as well as evidence from several other studies, has demonstrated that p53 can still suppress tumor formation in the absence of its apoptotic and growth arrest functions (Brady et al., 2011; Li et al., 2012; Valente et al., 2013). Preservation of p53-mediated metabolic and non-canonical target regulation in the $p53^{3KR}$ mouse model appears to contribute to tumor suppression, which no longer comes as a surprise, given the plethora of metabolic functions regulated by p53 (Liu et al., 2015; Maddocks and Vousden, 2011). Repression of *SLC7A11* by wild-type p53 and $p53^{3KR}$ was recently implicated in p53-mediated ferroptotic cell death and subsequent tumor suppression (Jiang et al., 2015a). A further loss of K98 acetylation on mouse p53, in addition to the loss of other acetylation events, disrupted its regulation on *SLC7A11*, therefore abrogating its ability to induce ferroptosis. A recent report characterizing a tumor-prone S47 p53 mutant showed that the majority of the downstream effectors that were compromised by the single amino acid mutation are involved in ferroptosis and metabolism, thus further corroborating our findings that ferroptosis may be inextricably linked to p53-mediated tumor suppression (Jennis et al., 2016).

Loss of *TIGAR* regulation with $p53^{4KR98}$ may also theoretically contribute to impairment of p53-mediated tumor suppression, although functional study of TIGAR-knockout mice suggests a tumor pro-survival role for the TIGAR gene (Cheung et al., 2013). There may also be other p53 targets (identified or yet unknown) regulated by $p53^{3KR}$, of which regulation is compromised in the absence of K98 acetylation, that played a part in the dynamics of tumor suppression observed in our study. In conclusion, we have identified a mouse p53 acetylation at K98 lysine (K101 for human p53) that is crucial for the regulation of p53 metabolic targets, and the loss of K98 acetylation in the absence of other critical acetylations in the DNA domain resulted in the failure of p53 to mediate ferroptosis and effectively suppress tumor growth through these targets.

EXPERIMENTAL PROCEDURES

Protein Purification and Mass Spectrometry

To purify the acetylated p53 protein for mass spectrometric analysis, H1299 cells were co-transfected with CMV-Flag-p53 and CMV-CBP-HA, cultured for 16 hr, and then treated with 1 μM TSA + 5 mM nicotinamide for 8 hr. Cells were harvested and lysed in the Flag-lysis buffer (50 mM Tris-HCl [pH 7.9], 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% sarkosyl, 10% glycerol, and fresh proteinase inhibitor cocktail; Sigma) plus 2 μM TSA and 10 mM nicotinamide. The cell extracts were then immunoprecipitated with the anti-Flag monoclonal antibody-conjugated M2 agarose beads (Sigma) and eluted using Flag peptide (Sigma). The eluted material was resolved by SDS-PAGE on a 4%–20% Tris-glycine gradient gel (Invitrogen), and the p53 bands were excised and subjected to mass spectrometric analysis.

Cell Death Assay

For cell death assays, p53 was pre-activated for 24 hr by doxycycline in Tet-on-inducible stable line cells, followed by treatment with or without erastin for an additional 48 hr. Cells were approximately 40% confluent when erastin was added to the culture. For quantification of cell death, cells were trypsinized and stained with trypan blue, followed by counting with a hemocytometer using standard protocol. Cells stained blue were considered as dead cells.

Mouse Xenograft

H1299 Tet-on 3KR and 4KR98 stable cell lines were treated with or without doxycycline (0.5 μg/mL) for 40 hr. Cells (1.2×10^6/100 μL) were then mixed with Matrigel (BD Biosciences) at 1:1 ratio (volume) and injected subcutaneously into Nude mice (Nu/Nu; Charles River). Mice were fed either with control food or food containing doxycycline hyclate (Harlan; 625 mg/kg). Six weeks after injection, mice were euthanized and tumors were dissected from under the skin. Maintenance and experimental procedures of mice were approved by the Institutional Animal Care and Use Committee (IACUC) of Columbia University.

Statistical Methods

Statistical analysis was performed using Excel. Treatment samples were compared using the two-sample Student's t test. Data were presented as means ± SEM. Statistical significance was defined as $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.022.

AUTHOR CONTRIBUTIONS

The whole project was conceived and designed by S.-J.W. and W.G. Experiments were performed mainly by S.-J.W., D.L., and Y.O. The mass spectrometry analysis was performed by Y.C. and Y.Z. Some of the experiments were performed with help from L.J. The paper was written by S.-J.W., D.L., and W.G.

ACKNOWLEDGMENTS

This work was supported by the National Cancer Institute of the NIH under award 5RO1CA172023, 5RO1CA190477, and 5RO1CA085533 to W.G. and RO1GM105933 and RO1GM115961 to Y.Z. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Received: March 3, 2016
Revised: August 1, 2016
Accepted: September 8, 2016
Published: October 4, 2016

REFERENCES

Brady, C.A., Jiang, D., Mello, S.S., Johnson, T.M., Jarvis, L.A., Kozak, M.M., Kenzelmann Broz, D., Basak, S., Park, E.J., McLaughlin, M.E., et al. (2011). Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. Cell *145*, 571–583.

Cheung, E.C., Athineos, D., Lee, P., Ridgway, R.A., Lambie, W., Nixon, C., Strathdee, D., Blyth, K., Sansom, O.J., and Vousden, K.H. (2013). TIGAR is required for efficient intestinal regeneration and tumorigenesis. Dev. Cell *25*, 463–477.

Choudhury, A.R., Ju, Z., Djojosubroto, M.W., Schienke, A., Lechel, A., Schaetzlein, S., Jiang, H., Stepczynska, A., Wang, C., Buer, J., et al. (2007). Cdkn1a

deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. Nat. Genet. *39*, 99–105.

Dixon, S.J., Lemberg, K.M., Lamprecht, M.R., Skouta, R., Zaitsev, E.M., Gleason, C.E., Patel, D.N., Bauer, A.J., Cantley, A.M., Yang, W.S., et al. (2012). Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell *149*, 1060–1072.

Eischen, C.M., and Lozano, G. (2014). The Mdm network and its regulation of p53 activities: a rheostat of cancer risk. Hum. Mutat. *35*, 728–737.

Feng, Z., Lin, M., and Wu, R. (2011). The regulation of aging and longevity: a new and complex role of p53. Genes Cancer *2*, 443–452.

Gannon, H.S., and Jones, S.N. (2012). Using mouse models to explore MDM-p53 signaling in development, cell growth, and tumorigenesis. Genes Cancer *3*, 209–218.

Gao, M., Monian, P., Quadri, N., Ramasamy, R., and Jiang, X. (2015). Glutaminolysis and transferrin regulate ferroptosis. Mol. Cell *59*, 298–308.

Helton, E.S., and Chen, X. (2007). p53 modulation of the DNA damage response. J. Cell. Biochem. *100*, 883–896.

Jennis, M., Kung, C.P., Basu, S., Budina-Kolomets, A., Leu, J.I., Khaku, S., Scott, J.P., Cai, K.Q., Campbell, M.R., Porter, D.K., et al. (2016). An African-specific polymorphism in the TP53 gene impairs p53 tumor suppressor function in a mouse model. Genes Dev. *30*, 918–930.

Jiang, L., Kon, N., Li, T., Wang, S.J., Su, T., Hibshoosh, H., Baer, R., and Gu, W. (2015a). Ferroptosis as a p53-mediated activity during tumour suppression. Nature *520*, 57–62.

Jiang, L., Hickman, J.H., Wang, S.J., and Gu, W. (2015b). Dynamic roles of p53-mediated metabolic activities in ROS-induced stress responses. Cell Cycle *14*, 2881–2885.

Kruse, J.P., and Gu, W. (2009). Modes of p53 regulation. Cell *137*, 609–622.

Levine, A.J., and Oren, M. (2009). The first 30 years of p53: growing ever more complex. Nat Rev Cancer *9*, 749–758.

Li, T., Kon, N., Jiang, L., Tan, M., Ludwig, T., Zhao, Y., Baer, R., and Gu, W. (2012). Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. Cell *149*, 1269–1283.

Liu, J., Zhang, C., Hu, W., and Feng, Z. (2015). Tumor suppressor p53 and its mutants in cancer metabolism. Cancer Lett. *356* (*2 Pt A*), 197–203.

Maddocks, O.D., and Vousden, K.H. (2011). Metabolic regulation by p53. J. Mol. Med. *89*, 237–245.

Mellert, H.S., and McMahon, S.B. (2009). Biochemical pathways that regulate acetyltransferase and deacetylase activity in mammalian cells. Trends Biochem. Sci. *34*, 571–578.

Michalak, E.M., Villunger, A., Adams, J.M., and Strasser, A. (2008). In several cell types tumour suppressor p53 induces apoptosis largely via Puma but Noxa can contribute. Cell Death Differ. *15*, 1019–1029.

Sykes, S.M., Mellert, H.S., Holbert, M.A., Li, K., Marmorstein, R., Lane, W.S., and McMahon, S.B. (2006). Acetylation of the p53 DNA-binding domain regulates apoptosis induction. Mol Cell *24*, 841–851.

Tang, Y., Luo, J., Zhang, W., and Gu, W. (2006). Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. Mol Cell *24*, 827–839.

Tang, Y., Zhao, W., Chen, Y., Zhao, Y., and Gu, W. (2008). Acetylation is indispensable for p53 activation. Cell *133*, 612–626.

Valente, L.J., Gray, D.H., Michalak, E.M., Pinon-Hofbauer, J., Egle, A., Scott, C.L., Janic, A., and Strasser, A. (2013). p53 efficiently suppresses tumor development in the complete absence of its cell-cycle inhibitory and proapoptotic effectors p21, Puma, and Noxa. Cell Rep. *3*, 1339–1345.

Vousden, K.H., and Prives, C. (2009). Blinded by the light: the growing complexity of p53. Cell *137*, 413–431.

Wang, S.J., and Gu, W. (2014). To be, or not to be: functional dilemma of p53 metabolic regulation. Curr. Opin. Oncol. *26*, 78–85.

Zhang, Q., Zeng, S.X., Zhang, Y., Zhang, Y., Ding, D., Ye, Q., Meroueh, S.O., and Lu, H. (2012a). A small molecule Inauhzin inhibits SIRT1 activity and suppresses tumour growth through activation of p53. EMBO Mol. Med. *4*, 298–312.

Zhang, Y., Zhang, Q., Zeng, S.X., Zhang, Y., Mayo, L.D., and Lu, H. (2012b). Inauhzin and Nutlin3 synergistically activate p53 and suppress tumor growth. Cancer Biol. Ther. *13*, 915–924.